Functional interaction between $InsP_3$ receptors and store-operated Htrp3 channels

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Calcium ions are released from intracellular stores in response to agonist-stimulated production of inositol 1,4,5-trisphosphate ($InsP_3$), a second messenger generated at the cell membrane. Depletion of Ca^{2+} from internal stores triggers a capacitative influx of extracellular Ca^{2+} across the plasma membrane[1,2]. The influx of Ca^{2+} can be recorded as store-operated channels (SOC) in the plasma membrane or as a current known as the Ca^{2+}-release-activated current (I_{crac})[3–5]. A critical question in cell signalling is how SOC and I_{crac} sense and respond to Ca^{2+}-store depletion: in one model, a messenger molecule is generated that activates Ca^{2+} entry in response to store depletion[1,6]; in an alternative model[7], $InsP_3$ receptors in the stores are coupled to SOC and I_{crac}. The mammalian Htrp3 protein[8] forms a well defined store-operated channel[8,9] and so provides a suitable system for studying the effect of Ca^{2+}-store depletion on SOC and I_{crac}. We show here that Htrp3 channels stably expressed in HEK293 cells are in a tight functional interaction with the $InsP_3$ receptors. Htrp3 channels present in the same plasma membrane patch can be activated by Ca^{2+} mobilization in intact cells and by $InsP_3$ in excised patches. This activation of Htrp3 by $InsP_3$ is lost on extensive washing of excised patches but is restored by addition of native or recombinant $InsP_3$-bound $InsP_3$ receptors. Our results provide evidence for the coupling hypothesis[7], in which $InsP_3$ receptors activated by $InsP_3$ interact with SOC and regulate I_{crac}.

A typical response of control HEK293 cells to application of Ca^{2+}-mobilizing agonists is the activation of a miniature Ca^{2+} channel (Fig. 1a). As the properties of this channel are similar to those described for miniature channels in several cell types[10,11], it may be the native store-operated channel of HEK293 cells. Under the same conditions, the majority (124 out of 155) of Htrp3-expressing cells (T3 cells) respond by activation of a large, non-selective channel (Fig. 1b, c) that was seen only in T3 cells (Fig. 2a) and had the same properties as Htrp3. In agreement with earlier results[9,12,13], the channel had a major conductance of 66 pS (Fig. 2b), but from the previous results[9,12,13] and those shown in Fig. 1, together with all-point amplitude histograms in Fig. 2f and noise analysis (data not shown), Htrp3 also has a 17-pS conductance (Fig. 2b, d–f). Both states have similar kinetic parameters (Fig. 2) and were almost equally prevalent in most records (Fig. 2d). Htrp3 had the same permeability for K^+, Na^+, Ca^{2+} and Ba^{2+}, and its open probability increased at membrane potentials above +40 mV (Fig. 2c). The 17-pS channels may assemble into a 66-pS complex, or the 66-pS channel may have a 17-pS substate; these possibilities are not investigated here.

A central finding is that Htrp3 can be activated in isolated, cell-free plasma membrane patches by application of $InsP_3$ to the cytosolic face of a patch. Excision of the membrane patch containing active Htrp3 caused a loss of channel activity, which could then be restored by 2 μM $InsP_3$ (Fig. 1b). The kinetic properties of channels activated by agonist in intact cells or by $InsP_3$ in excised patches were the same, including conductance, ion selectivity, voltage-dependence of the open probability (NP_o) and mean open time (Fig. 2). Furthermore, an inhibitor of SOC/I_{crac} (ref. 14) and Htrp3 channels[15], SKF 96265 (SKF), inhibited the channels activated by $InsP_3$ in excised patches (Fig. 3a). The effect of $InsP_3$ on Htrp3 was inhibited by heparin (Fig. 3b) and by the membrane-permeable inhibitor of the $InsP_3$ receptor ($InsP_3R$), xestospongin C (Xest C; Fig. 3c)[16]. Considering that $InsP_3$ was the only second messenger present under excised-patch conditions, the results shown in Figs 1–3 indicate that $InsP_3$ receptors communicate with Htrp3 and regulate its activity, in agreement with the coupling hypothesis.

The dependence of Htrp3 activity on the Ca^{2+} content of internal stores was tested by using thapsigargin (Tg), which depletes stored Ca^{2+} at the $InsP_3$ concentrations present in resting cells[17,18]. Thapsigargin-induced store depletion in intact cells and cytosolic $InsP_3$ in

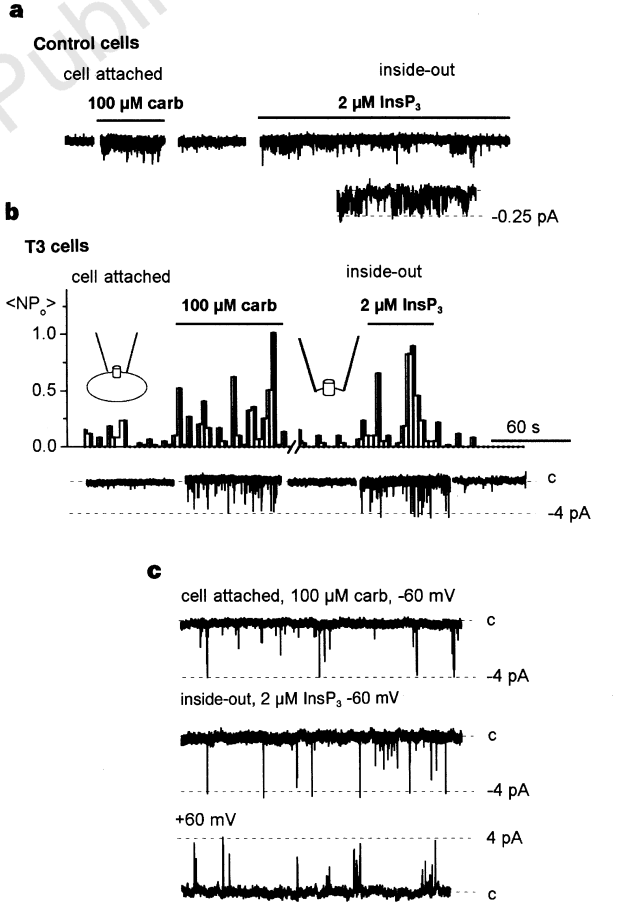

Figure 1 Activation of Htrp3 channels by Ca^{2+}-mobilizing agonists and $InsP_3$. The cell-attached mode of the patch-clamp technique was used to monitor channel activity in plasma membrane patches of intact control HEK293 and Htrp3-expressing cells (T3 cells) and in membrane patches excised from them. **a**, Ca^{2+} current in the control cell was recorded at a holding potential of –60 mV. As indicated, the cell was stimulated with 100 μM carbachol (carb) and subsequently the patch was excised and exposed to 2–10 μM $InsP_3$. Bottom right, a segment of the $InsP_3$-activated current on an expanded timescale. **b**, The same protocol was used to record the activity of Htrp3 in T3 cells. The models indicate the experimental protocol. **c**, Htrp3 channel activity at an expanded timescale; the letter 'c' denotes the closed state, and the dashed lines represent channel openings to 66-pS state.

excised patches activated Htrp3 present in the same plasma membrane patch (Fig. 4a, b). Thapsigargin was not as effective as agonists in stimulating Htrp3, suggesting that both depleted stores and $InsP_3$-bound $InsP_3$ receptors are required for the activation of Htrp3 (see below). In agreement with this suggestion, we found that stimulation of thapsigargin-treated cells with agonist, which increases the cytosolic $InsP_3$ concentration after store depletion, further stimulated Htrp3 (Fig. 4c). These results are equivalent to the increased Ca^{2+} and Ba^{2+} influx that occurs in thapsigargin-treated T3 cells stimulated with carbachol[15].

The relation between the activities of the $InsP_3$ receptor and Htrp3 is confirmed by the requirement of an active $InsP_3$ receptor for activation of Htrp3. Figure 5a shows that irreversible depletion of internal stores by treatment of T3 cells with agonist and thapsigargin caused a persistent activation of Htrp3. Inhibition of channel activity by addition of 20 μM SKF (Fig. 5a, c) to the bath indicated that the current was carried by Htrp3. Diffusion of heparin into the cytosol after Htrp3 activation effectively inhibited Htrp3 activity, despite the continued presence of thapsigargin which maintains the stores in a depleted state (Fig. 5b). In the cell-attached mode, Htrp3 was activated by thapsigargin in the absence (Fig. 5d, f) or presence (Fig. 5e, f) of agonist. Incubation of these cells with Xest C markedly inhibited Htrp3 activity. These results indicate that an active $InsP_3$ receptor is required for maintaining Htrp3 in the active state.

The functional coupling between $InsP_3$ receptors and Htrp3 suggests that stable expression of Htrp3 should be reflected in a concomitant change in the number of $InsP_3$ receptors. Figure 6a shows that expression of Htrp3 increased the expression of all $InsP_3$ receptor isoforms in HEK293 cells. These findings can explain why cells transiently transfected with large amounts of Htrp3 (~600 channels per cell) show high spontaneous activity[13], whereas in T3 cells (~20 Htrp3 channels per cell) the spontaneous activity of Htrp3 is low. It is likely that T3 cells adjust to the stable expression of Htrp3 by upregulating their expression of $InsP_3$ receptors.

The Htrp3 and $InsP_3$ receptors did not coimmunoprecipitate and neither could $InsP_3$ activate Htrp3 if the patches were extensively washed before application of $InsP_3$ (Fig. 6b). This suggests that there was a weak interaction between the channels. But we were able to reconstitute the Htrp3–$InsP_3$R complex by adding native or recombinant $InsP_3$ receptors to the patches. We used rat or bovine cerebellar microsomes as a source of native $InsP_3$ receptors[19], and rat forebrain microsomes, which are a poor source of $InsP_3$ receptors[19], as a control. Addition of cerebellar microsomes to the solution containing $InsP_3$ strongly activated Htrp3, whereas omission of $InsP_3$ or microsomes caused channel activity to drop to zero (Fig. 6). We were able to restore Htrp3 activity in ~25% of experiments, suggesting that targeting of the Htrp3 channel by the $InsP_3$ receptor is a stochastic event and depends on how a microsome binds to the patch. Persistent Htrp3 reactivation occurred in 3/12 experiments (Fig. 6b), and in 3/12 experiments the microsomes only transiently restored Htrp3 activity (Fig. 6d), as though binding was unstable. In 6/12 attempts, Htrp3 was not

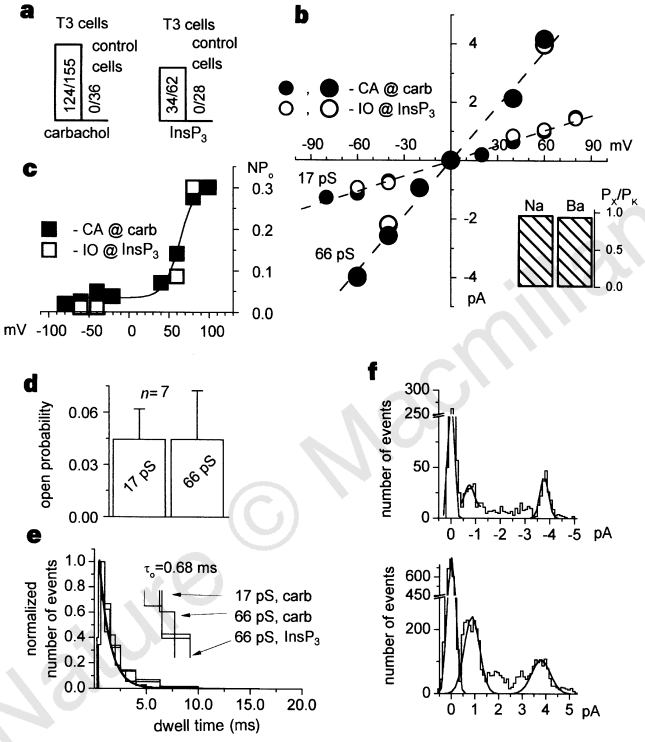

Figure 2 Kinetic properties of the channels activated by agonists in intact cells and by $InsP_3$ in excised patches. **a**, Summary of Htrp3 occurrence in T3 and control cells stimulated with carbachol in cell-attached and with $InsP_3$ in inside-out mode. **b**, The current–voltage relation for agonist (filled circles) and $InsP_3$-activated (open circles) channels (each point represents data from 2–7 experiments); large symbols, 66-pS conductance; small symbols, 17-pS conductance. Error bars (s.e.m.) are smaller than symbol size. Inset, relative permeabilities (P_x, calculated from reversal potentials) of the channels for Ba^{2+}, Na^+ and K^+. *I–V* curves for Ba^{2+} (not shown) were derived from 4 experiments. CA@carb, cell-attached and carbachol; IO@$InsP_3$, inside-out and $InsP_3$; P_K, K^+ permeability. **c**, A plot of Htrp3 NP_0 activated by agonist (filled squares) or $InsP_3$ (open squares) as a function of membrane potential. **d**, Probability of Htrp3 channel being open to 17-pS and 66-pS states. Data were obtained from 100–300-s current recordings. **e**, The dwell-time distributions for the 66-pS conductance activated by carbachol or $InsP_3$ and the 17-pS conductance activated by carbachol are superimposed. Data were normalized to facilitate comparison between the different conditions. The average dwell times were calculated from single exponential fits (thick line). The line represents fits for all three curves. **f**, All-point amplitude histograms were obtained at –60 (top) and +60 (bottom) mV from a representative record of Htrp3 activity. Note the presence of the two peaks corresponding to 17 and 66 pS. Peaks were fitted by gaussian distributions.

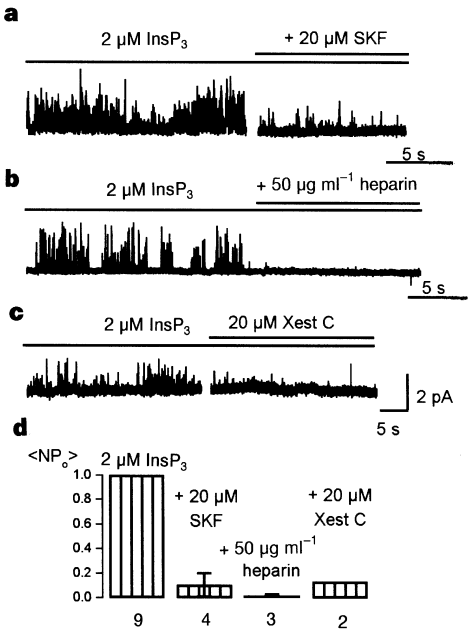

Figure 3 The effect of Htrp3 and $InsP_3$R channel inhibitors on $InsP_3$-induced activation. The experiments were done with inside-out patches. In all experiments, *I–V* relations were determined before and after application of the indicated inhibitor to identify the channels. **a**, SKF and **b**, heparin were added to the bath solution; **c**, Xest C was applied by puffing from an adjacent pipette. **d**, Summary of results from the number of experiments shown below the graph. NP_0 was averaged from 30–60 s current recordings before and after application of inhibitors.

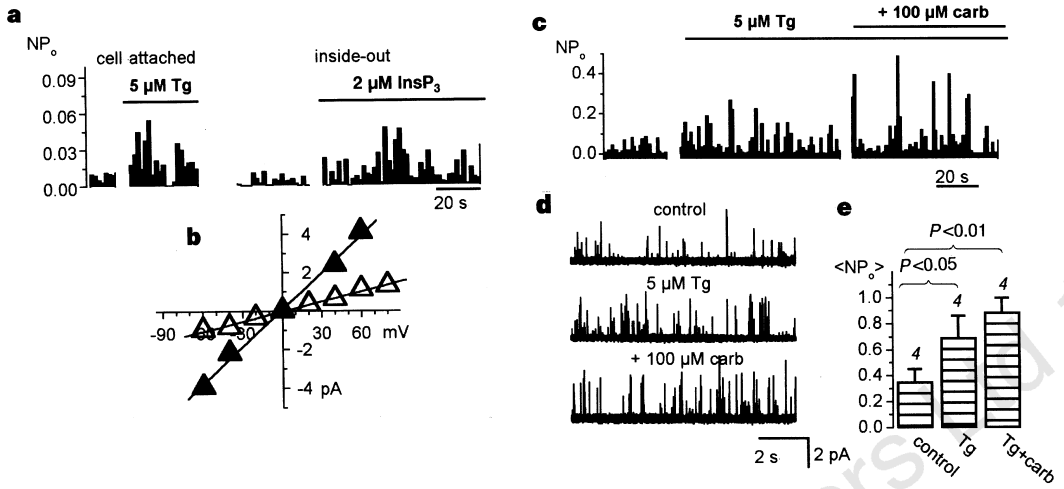

Figure 4 Activation of Htrp3 channels by thapsigargin and $InsP_3$. **a**, The protocol used for Fig. 1 was also used to study Htrp3 activation by Tg and $InsP_3$. **b**, *I–V* curve for Tg-induced channel activity. Each point represents the results of 2–6 experiments. Error bars (s.e.m.) are smaller than the symbol size. Slopes equal 16.8 pS (triangles) and 65 pS (filled triangles). **c**, NP_0 of Htrp3 induced by Tg and subsequent stimulation of the same cell by carbachol. **d**, Expanded segments of the current recording shown in **c**. **e**, Summary of results of the 4 experiments.

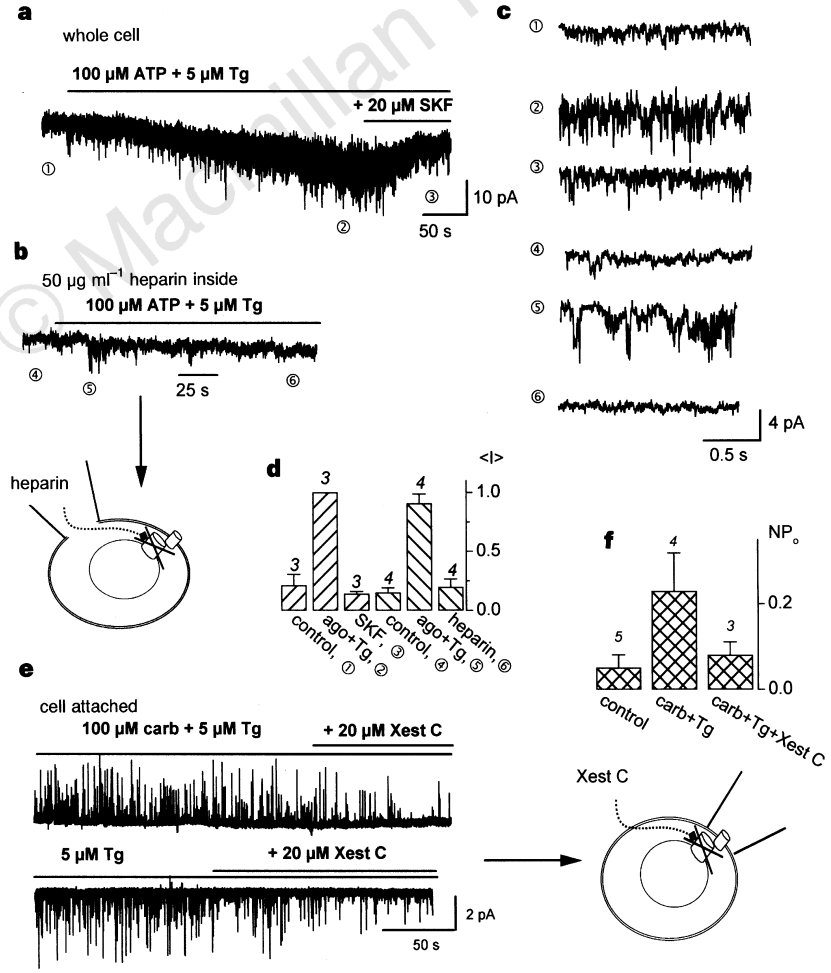

Figure 5 Suppression of Htrp3 activity by inhibitors of $InsP_3R$ in intact cells. **a–d**, The whole-cell mode of the patch-clamp technique was used to monitor channel activity. Htrp3 channels were activated by agonist and Tg in the **a**, absence, or **b**, presence of heparin in the pipette solution. In **a**, the indicated channels were inhibited by SKF. **c**, Segments of channel recordings (numbered) from the experiments in **a** and **b** at an expanded timescale. Experiments are summarized in **d**. Conditions and periods of estimation of channel activity are listed below the bars. In **e** and **f**, Htrp3 activity was monitored in cell-attached mode. Where indicated in **e**, cells treated with Tg in the presence (top) and absence (bottom) of carbachol were exposed to Xest C.

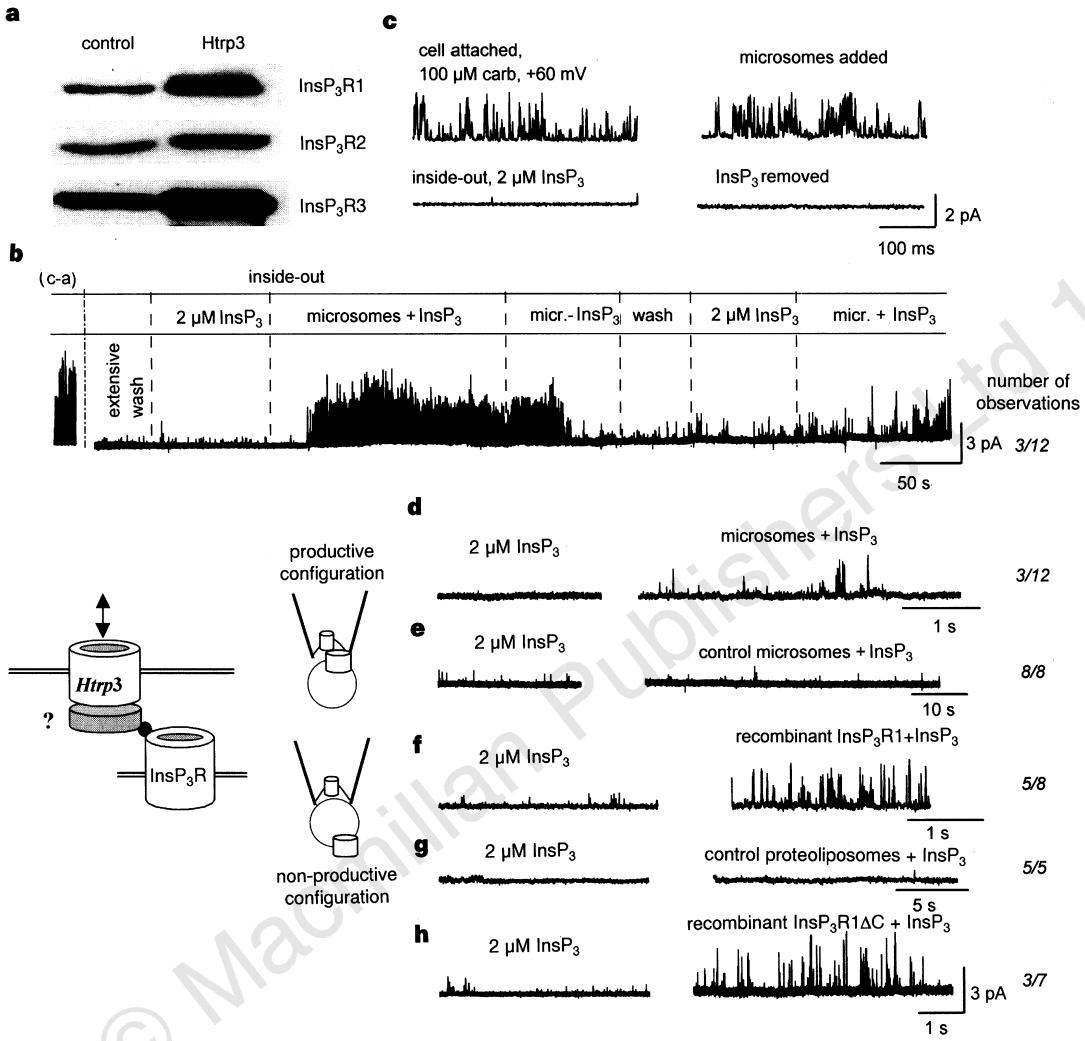

Figure 6 Reconstruction of Htrp3–$InsP_3$ receptor complexes. **a**, Western blot analysis of $InsP_3R$ expression in control and T3 cells, performed as before[26]. **b–h**, The protocol used for Fig. 1 was used to find the effect of **b–d**, cerebellar and **f–h**, recombinant $InsP_3R$ on Htrp3 activity. Initially, the currents were recorded in the cell-attached (c–a) mode to ensure that the patch contained the Htrp3 channel. The channel was then completely inactivated by patch excision and extensive washes until application of $InsP_3$ evoked no channel openings. The addition of bovine cerebellar microsomes (**b–d**) caused robust and stable (**b**, **c**) or transient (**d**) activation of Htrp3 channel. Removal of $InsP_3$ or microsomes inactivated the Htrp3 channels (**b**, **c**). The numbers of successful attempts are listed in the right column. **c**, Segments of current recordings at an expanded timescale from those in **b**. **c**, **g**, Typical experiments with control microsomes. **f**, **h**, Examples of activation of Htrp3 by recombinant $InsP_3R$ reconstituted into liposomes (**f**) and microsomes prepared from HEK293 cells expressing a C-terminally truncated $\Delta CInsP_3R1$ (**h**). Models on the left illustrate the proposed interactions under our experimental conditions.

activated, possibly because of impaired access of the $InsP_3$ receptor to a patch capped by a microsome free of $InsP_3$ receptors (see model in Fig. 6). Recombinant $InsP_3R1$, which had been partially purified on a sucrose gradient and reconstituted into liposomes, could also activate the Htrp3 channel when added together with $InsP_3$ to inside-out patches (5/8 attempts; Fig. 6f). Neither control forebrain microsomes (8/8 attempts) nor control proteoliposomes (5/5 attempts) activated Htrp3 channels (Fig. 6e, g).

The activation of Htrp3 by $InsP_3R1$ appears to contradict an earlier report[20] that deletion of all known isoforms of the $InsP_3$ receptor does not prevent stimulation of Ca^{2+} influx by thapsigargin. However, in these experiments[20] native $InsP_3$ receptors were replaced with truncated constructs: for example, only 100 amino acids of the C terminus of $InsP_3R1$ were deleted. Although this inactivated $InsP_3R1$ channel activity, it may have not prevented regulation of Ca^{2+} influx by $InsP_3R1$. To test this idea, we removed 154 amino acids from the C terminus of $InsP_3R1$ ($\Delta CInsP_3R1$) and tested its activity. Figure 6h shows that $\Delta CInsP_3R1$ was as effective as full-length $InsP_3R1$ in activating Htrp3. Furthermore, it is likely that the cells, which were triple mutants for $InsP_3$ receptors[20], expressed other Ca^{2+}-releasing channels, such as ryanodine receptors, which mediated the activation of Ca^{2+} influx by thapsigargin.

We have used molecular and functional approaches to show that activation of Htrp3 channels requires both Ca^{2+} depletion from internal stores and $InsP_3$ receptors occupied with $InsP_3$. The stimulation of Htrp3 activity by agonist in thapsigargin-treated cells and its inhibition by inhibitors of $InsP_3$ receptors in intact cells indicate that, in addition to Ca^{2+}-store depletion, the binding of $InsP_3$ to its receptor is required for Htrp3 activation; this conclusion is supported by the activation by $InsP_3$ of Htrp3 in excised plasma membrane patches. The loss of this activation in extensively washed patches and its restoration after incubating the patches with native or recombinant $InsP_3$ receptor suggests that the two channels interact either directly or indirectly. The regulation of Htrp3 by stored Ca^{2+} by a mechanism similar to that used to regulate I_{crac}, together with a recent demonstration of an I_{crac}-like current in excised patches that is activated by $InsP_3$ (refs 10, 11), suggests that the interaction between plasma membrane channels and $InsP_3$ receptors is used generally for regulating

store-dependent Ca^{2+} influx. Such regulation is reminiscent of excitation–contraction coupling in skeletal muscle[21,22]. Like the preferred localization of ryanodine receptors in terminal cisternae[22], microdomains of highly expressed $InsP_3$ receptors next to the plasma membrane[23] may form a site of regulatory interaction between the $InsP_3$ receptors and Ca^{2+}-influx channels. Irrespective of the nature of the interaction between the channels, our findings strongly support the coupling hypothesis[2,7]. □

Methods

Cells. Control cells and clone 56 of HEK293 cells stably expressing Htrp3 (ref. 8) were grown in DMEM medium supplemented with 10% FBS, 1% penicillin/streptomycin and 200 $\mu g\,ml^{-1}$ G418 (Gibco BRL) and maintained at 37 °C in 5% CO_2. One day before the experiments, cells were transferred to glass coverslips for patch-clamp recordings.

Electrophysiology. In the cell-attached mode, the pipette solution contained 100 mM Na/HEPES and 2 mM $CaCl_2$; pH of all solutions was 7.4. Before changing to high-K^+ solution, cells were bathed in normal Ringer solution containing (in mM): 140 NaCl, 10 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES. After a seal was established, the medium was replaced with a high-K^+ solution containing (in mM): 145 KCl, 5 NaCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES. For estimation of channel permeability for Ba^{2+}, pipettes were filled with 100 mM $BaCl_2$, 10 HEPES, or 100 mM Ba^{2+}/HEPES, 2 $BaCl_2$. Patches were excised into a solution containing (in mM): 140 potassium gluconate, 10 HEPES, 2 EGTA, 1 $MgCl_2$, 5 NaCl, 1.13 $CaCl_2$ (pCa7), or a solution without $CaCl_2$. For whole-cell studies, the pipette solution contained (in mM): 120 potassium gluconate, 20 HEPES, 10 EGTA, 1 $MgCl_2$, 5 NaCl, 5 ATP. Bath solution during whole-cell experiments was normal Ringer. Current was recorded with an Axopatch 200B patch-clamp amplifier equipped with a Digidata 1200 PC interface. Currents were recorded at 2–5 kHz, filtered at 0.2–1 kHz and analysed with Axoscope 1.1, PClamp 6.0.3 (Axon Instruments) and Origin 5.0 (Microcal) software. NP_0 was determined using the following equation: $NP_0 = \langle I \rangle / i$, where $\langle I \rangle$ and i are the mean channel current and unitary current amplitude, respectively.

Cerebellar microsomes. Bovine or rat cerebella (or forebrains) were homogenized in a solution containing (in mM): 50 Tris, 100 NaCl and 2 EDTA. The homogenate was centrifuged for 10 min at 500*g* and the supernatant was collected and centrifuged for 25 min at 40,000*g*. The pellet was resuspended in the bath solution during patch-clamp experiments and stored at −80 °C.

Expression of recombinant $InsP_3R$ and preparation of proteoliposomes. COS-1 or HEK293 cells were transfected with the plasmid p$InsP_3$R-T1 (recombinant $InsP_3R$), p(1-6)$InsP_3$R-T1 ($\Delta CInsP_3R$), or salmon sperm single-stranded DNA (control in Fig. 6g) as described in ref. 24. Cells were collected 48–72 h post-transfection and microsomes were prepared as described[24]. Microsomes from control and $InsP_3$R1-expressing cells were solubilized in 1% CHAPS buffer and fractionated through 5–20% sucrose (w/v) gradients. Gradient fractions containing $InsP_3R$ were identified by immunoblotting with $InsP_3$R1-specific antibody. Fresh $InsP_3R$ fractions and parallel fractions of control cells were reconstituted into liposomes as described[25]. Proteoliposomes from cells transfected with p$InsP_3$R-T1 yielded pronounced channel activity when incorporated into lipid bilayers, and those containing the COS-1 control had no channel activity (data not shown). HEK293 cells expressing $\Delta CInsP_3R1$ were used to prepare microsomes by the procedure already described for the preparation of cerebellar microsomes.

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The receptor Msn5 exports the phosphorylated transcription factor Pho4 out of the nucleus

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The movement of many transcription factors, kinases and replication factors between the nucleus and cytoplasm is important in regulating their activity[1]. In some cases, phosphorylation of a protein regulates its entry into the nucleus[2]; in others, it causes the protein to be exported to the cytoplasm[3–6]. The mechanism by which phosphorylation promotes protein export from the nucleus is poorly understood. Here we investigate how the export of the yeast transcription factor Pho4 is regulated in response to changes in phosphate availability. We show that phosphorylation of Pho4 by a nuclear complex of a cyclin with a cyclin-dependent kinase, Pho80–Pho85, triggers its export from the nucleus. We also find that the shuttling receptor used by Pho4 for nuclear export is the importin-β-family member Msn5 (refs 7, 8), which is required for nuclear export of Pho4 *in vivo* and binds only to phosphorylated Pho4 in the presence of the GTP-bound form of yeast Ran *in vitro*. Our results reveal a simple mechanism by which phosphorylation can control the nuclear export of a protein.

When yeast are grown in phosphate-rich medium, the transcription factor Pho4 is phosphorylated by the Pho80–Pho85 cyclin–cyclin-dependent kinase (CDK) complex[9] and is localized to the cytoplasm[10], thereby turning off transcription of genes that are turned on specifi-